

STIC-ILL

From: Schmidt, Mary
Sent: Thursday, December 12, 2002 2:48 PM
To: STIC-ILL
Subject: last set of references 09/122,588

Mu
RC261-A/C2

VEGF references:

Thierry et al., Proceedings of the american association for cancer research annual meeting, 36, 0, p413, 1995.

Yung et al., neurology 48, 3 suppl., 2, pa22, 1997.

ke et al., proceedings of the american association for cancer research annual meeting, 37, 0, p60, 1996.

ellis eta l., surgery, nov. 1996, 120, 5, p871-8.

martiny-baron et al., current opinion in biotechnology, dec. 1995, 6, 6, p675-80.

warren et al., journal of clinical investigation, apr. 1995, 95, 4, p1789-97.

o-brien et al., cancer research , feb. 1, 1995, 55, 3, p510-3.

chaudhary et al., molecular biology of the cell, 7, suppl., p352a, 1996.

saleh et al, cancer research, 1-15-1996, 56, 2, 393-401.

claffey et al, cancer research, 1-1-1996, 56, 1, 172-81.

levy et al, j. of biological chem, 10-11-1996, 271, 41, p25492-7.

thanks,
Melissa
11e12 mailboxes

Inhibition of Growth of C6 Glioma Cells *in Vivo* by Expression of Antisense Vascular Endothelial Growth Factor Sequence¹

Mary Saleh,² Steven A. Stacker, and Andrew F. Wilks

Neuroscience Center, Department of Surgery, The University of Melbourne, Grattan Street [M. S.], and Growth Regulation Laboratory, Ludwig Institute for Cancer Research, Melbourne Tumor Biology Branch, P.O. Box 2008 [S. A. S., A. F. W.], Parkville Victoria 3050, Australia

ABSTRACT

Tumor angiogenesis involves a combination of events including the production of inhibitors, proteases, and angiogenic factors that have a chemotactic and mitogenic effect on endothelial cells. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that promotes angiogenesis in solid tumors, including brain tumors such as astrocytomas. As an approach to the development of new strategies for gene therapy of brain tumors, we have interrupted the VEGF/VEGF receptor paracrine pathway in an attempt to inhibit angiogenesis and thereby control tumor growth. Rat C6 glioma cells were transfected with a eukaryotic expression vector bearing an antisense-VEGF cDNA. Stable transfectants were observed to express reduced levels of VEGF in culture under hypoxic conditions. When implanted s.c. into nude (*nu/nu*) mice, growth of the antisense-VEGF cell lines was observed to be greatly inhibited compared to control cells, despite the fact that they have a faster division time *in vitro*. Analysis of these tumors revealed that they have fewer blood vessels and a higher degree of necrosis, which is a plausible explanation for the reduced tumor size. We believe antisense-VEGF can be successfully used to control tumor growth and may provide the basis for the development of antiangiogenic gene therapy.

INTRODUCTION

VEGF³ is a powerful mitogen for vascular endothelial cells, both *in vitro* (1) and *in vivo* (2). In addition, VEGF has the property of inducing vascular permeability *in vivo*, hence its alternative name, vascular permeability factor (3). VEGF exists as a homodimer of approximately M_r 46,000, composed of one pair of four possible monomers, which appear to be generated by differential splicing of the VEGF gene transcript (1, 4). There are two known receptors for VEGF, both of which are protein tyrosine kinases. VEGF-R1, originally named FLT (5), and VEGF-R2 [also known as flk-1 (6), NYK (7), and KDR (8)], are structurally related to each other, and both belong to the so-called Class III protein tyrosine kinase receptors, along with the receptors for PDGF (9, 10). The ligands for these receptors are also structurally related, being members of the "cysteine knot" family of proteins to which PDGF and VEGF belong.

There is mounting evidence that VEGF and its receptors play pivotal roles in angiogenesis: that which occurs during normal embryonic development (6, 11) and that which also occurs as a consequence of the requirement of solid tumors to develop their own vascular beds (11). Three lines of evidence point to the central role played by VEGF in tumor angiogenesis: (a) the detection of high levels of VEGF expression in palisading cells around regions of necrosis in a number of solid tumor systems, coupled with the rapid induction of VEGF when tumor cells are grown under hypoxic con-

ditions (2, 12-14), links VEGF expression in tumor cells and a physiological stimulus (hypoxia) that might promote its expression. The detection of elevated levels of both of the receptors of VEGF in tumor blood vessels suggests that VEGF may be having an impact upon the growth of these tumor endothelial cells; (b) injection of antibodies against VEGF markedly reduce the *in vivo* growth of s.c. injected tumor cells, which are known to produce robust tumor angiogenesis (15); and (c) introduction into tumor endothelial cells of a dominant-negative version of the VEGF-R2 by means of retroviral transfer markedly reduced tumor size (16). Taken together, these data present a formidable case for the notion that VEGF and its receptors play an important role in the development of tumor vasculature and that interference of this paracrine circuit might provide an excellent opportunity for therapeutic intervention, particularly in situations where tumors might otherwise be difficult to treat.

Brain tumors are responsible for approximately 2% of all cancer deaths. Malignant glial neoplasms comprise 40-50% of brain tumor cases and are the fourth most significant form of malignancy in terms of life years lost (17). Astrocytomas are the most common type of human brain tumor and are classified according to malignancy as astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme. The progression of low to high grade is characterized by an increase in neovascularization, focal necrosis, and cellular proliferation. Current treatments, such as surgery and chemotherapy, only provide short-term management of the disease (17, 18). Hence, the development of new primary and adjuvant treatments for glioma is vital.

Analysis of human gliomas has shown that they are highly heterogeneous, although they may be of the same malignancy classification. Furthermore, the cellular profile of each individual tumor is also highly heterogeneous in that they contain cells that are at varying stages of malignancy and have growth factor/receptor expression profiles that can differ markedly (19, 20). Therefore, to develop an effective strategy of gene therapy for glioma, a process common to all the tumors must be targeted. As glioblastoma multiforme is one of the most highly vascularized solid tumors in terms of vasoproliferation, endothelial cell hyperplasia, and endothelial cell cytology (21) the process of angiogenesis represents a suitable target for gene therapy.

We have used the rat C6 glioma cell line, an established model for human glioma (22), to assess the feasibility of disrupting the VEGF/VEGF receptor pathway of angiogenesis by antisense-VEGF expression. The *in vivo* growth of C6 cell lines expressing antisense-VEGF was demonstrated to be significantly suppressed due to a decrease in the number of blood vessels within tumors and a subsequent increase in the degree of necrosis. The use of antisense-VEGF gene therapy presents an exciting avenue of research into new adjuvant therapies for human glioma and provides the basis for the further development of antiangiogenic gene therapy strategies.

MATERIALS AND METHODS

Antisense-VEGF Vector Construction. The mouse VEGF₁₆₅ cDNA of 650 bp was initially isolated from the mouse colon by PCR. The clone was demonstrated to encode a functional VEGF because it could induce vascular permeability in the Miles Assay and bind to VEGF-R2 in a receptor-binding

Received 8/15/95; accepted 11/13/95.

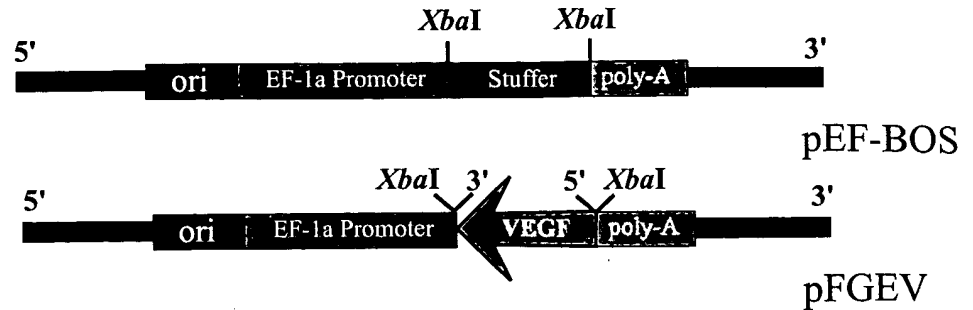
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ M. S. was supported by a National Health and Medical Research Council Australian Postdoctoral Fellowship. S. A. S. and A. F. W. acknowledge the support of the National Health and Medical Research Council.

² To whom requests for reprints should be addressed. Phone: 61-3-9342-7616; Fax: 61-3-9347-8332.

³ The abbreviations used are: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; NCS, newborn calf serum; RT-PCR, reverse transcription-PCR.

Fig. 1. Schematic representation of the anti-sense-VEGF cDNA subcloned into the pEF-BOS eukaryotic expression vector. The cDNA clone runs antisense relative to the EF-1 α promoter.



assay.⁴ The VEGF₁₆₅ cDNA was subcloned into the pEF-BOS eucaryotic expression vector (23) using the *Xba*I restriction enzyme site. Restriction enzyme analysis (*Eco*RI and *Pst*I) was used to confirm the antisense orientation of the VEGF cDNA in the pEF-BOS vector from individual transformants.

Rat C6 Glioma Culture Conditions. Rat C6 glioma cells (CCL 107; American Type Culture Collection, Rockville, MD) were routinely cultured in RPMI 1640/5% NCS (heat-inactivated) and 2 mM L-glutamine in a humidified atmosphere of 5% CO₂. Prior to calcium phosphate transfection, the cells were grown in DMEM/5% NCS and 2 mM L-glutamine with 10% CO₂.

Calcium Phosphate Transfection. Rat C6 cells were grown in DME/5% NCS in 10-cm plastic dishes to 60% confluence. The calcium phosphate transfection was performed as described previously (24) using 20 μ g of the antisense-VEGF construct DNA (or pEF-BOS vector alone) and 2 μ g of the neomycin-resistance (pgk-neo) plasmid. The cells were then subjected to glycerol shock (5 ml DME/10% NCS/15% glycerol) for 4 min at room temperature and then washed in culture medium and incubated as described. After 48 h or when the cells were confluent, they were trypsinized from the plates and replated at a dilution of 1:20 in selection medium containing Neomycin G418 (1.2 mg/ml). Cell death was observed after 5 days in culture, and discreet colonies were apparent by 7 days post-selection. Individual colonies were then isolated and grown in 24-well culture plates. Genomic DNA and total RNA were then isolated from these colonies, and PCR and RT-PCR analysis was performed. Clones demonstrated to express the antisense-VEGF construct were then recloned by growing single cells in 96-well plates and the RT-PCR analysis was repeated.

PCR Analysis. PCR was used to determine which rat C6 clones were successfully transfected with the antisense-VEGF construct or control vector construct. PCR was performed on genomic DNA isolated from rat C6 glioma cells and individual clones of transfected cells using a sense primer that corresponds to the 3' terminal sequence of the VEGF cDNA insert (5' GGG ATC CTC ACC GCC TCG GCT TGT CAC A-3') and an antisense primer that corresponds to the region of the polyadenylation sequence of the pEF-BOS cloning vector (5'-GTC CCA CGT GGG GAC CCT-3'). The PCR reaction was performed using standard protocols with 35 cycles of 1 min at 95°C and 2 min at 72°C. Clones demonstrated to be positive by this technique were then analyzed for their ability to express the antisense construct by RT-PCR. The RT-PCR reactions were performed on total RNA isolated from the positive clones using a reverse transcription system kit (Promega Corporation, Madison, WI) as described by the manufacturers with the same PCR primers as described above.

Southern Blot Analysis. Genomic DNA was isolated from cultured rat C6 glioma or transfected cells and then digested with the restriction enzyme *Eco*RI and electrophoresed on 1% agarose gels. The DNA was transferred onto Hybond N nylon membrane (Amersham Corp., Buckinghamshire, United Kingdom) and hybridized with the VEGF cDNA in 1 mM EDTA/0.5 M NaHPO₄ (pH 7.2) and 7% SDS at 65°C (25) for 16 h. The membranes were washed in 0.1 \times SSC/0.1% SDS at 65°C. cDNA probes were radiolabeled with [α -³²P]dCTP (Amersham) according to the random primer method (26).

Northern Blot Analysis. Total RNA was isolated from cultured rat C6 glioma and transfected cells using TRIZOL (Gibco BRL/Life Science Technologies, Inc., Gaithersburg, MD), essentially as described by the manufacturer, and is based on the guanidine isothiocyanate extraction method (27).

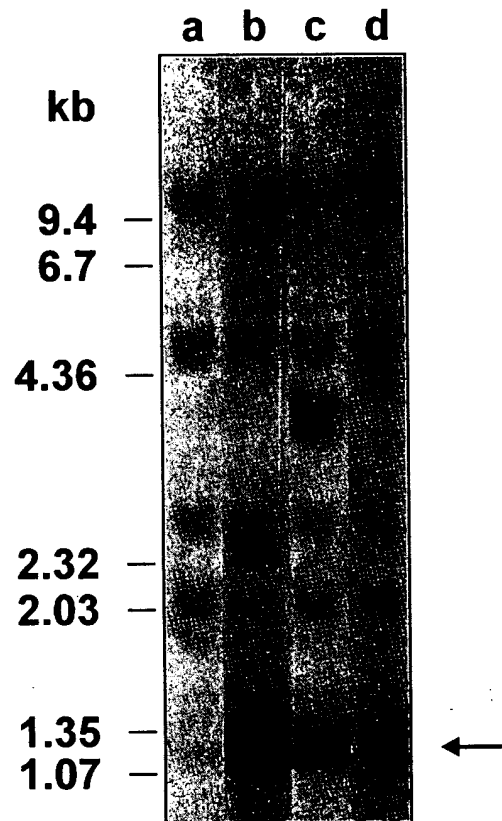


Fig. 2. Genomic Southern analysis of rat C6 glioma cells and C6 cells transfected with antisense-VEGF. Genomic DNA was digested with *Eco*RI and probed with the ³²P-VEGF cDNA. Lane a, rat C6 glioma cells; Lane b, rat C6 cells transfected with antisense-VEGF, clone anti-19; Lane c, anti-20; Lane d, anti-24. Arrow, bands representing integrated VEGF. Molecular weight markers are in kb.

Routinely, 20 μ g of total RNA were electrophoresed on 1% agarose/3% formaldehyde RNA gels and transferred onto Hybond N nylon membrane (Amersham). The filters were prehybridized in 1 mM EDTA/0.5 M NaHPO₄ (pH 7.2) and 7% SDS at 65°C (25) for 16 h. Hybridization was performed with the isolated VEGF cDNA in the above solution also at 65°C for 16 h. The filters were initially washed in 0.1 \times SSC/0.1% SDS at 55°C and then at a higher stringency of 0.1 \times SSC/0.1% SDS at 65°C. cDNA probes were radiolabeled with [α -³²P]dCTP (Amersham), according to the random primer method (26). Northern blots were also routinely hybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe as a control for the quantity of RNA loaded onto the gels.

Western Blot Analysis. Cell supernatants were collected from rat C6 cells or transfected cells grown in medium supplemented with 100 μ M CoCl₂ at 0, 4, 8, and 24 h. Supernatants were concentrated by heparin-Sepharose-CL4B (5 ml/100 μ l of beads; Pharmacia) by incubation at 4°C for 4–16 h with rotation. Following this, the beads were washed with 100 mM sodium phosphate (pH

⁴ S. A. Stacker and A. F. Wilks, unpublished data.

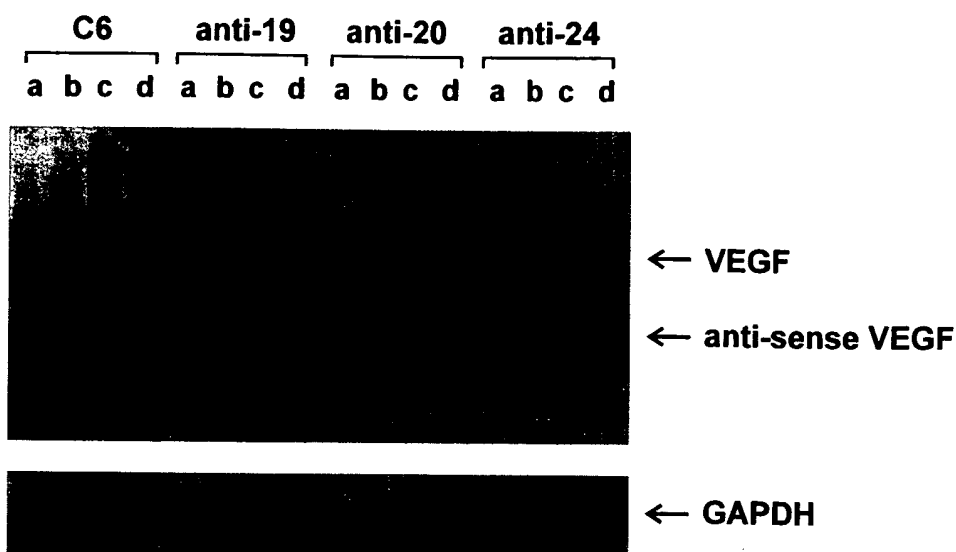


Fig. 3. Northern blot analysis of the expression of antisense-VEGF cDNA and the relative induction of endogenous VEGF in C6 glioma cells and the antisense-VEGF cell lines grown under hypoxic culture conditions. Cells were grown in culture medium supplemented with $100 \mu\text{M}$ CoCl_2 to simulate hypoxia, and total RNA was isolated at 0 h (a), 4 h (b), 8 h (c), and 24 h (d). Following hybridization with the VEGF cDNA, the membrane was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA control (lower panel).

7.2), and the beads then eluted with $2\times$ SDS-PAGE sample buffer and loaded directly onto a denaturing SDS-PAGE gel. After electrophoresis, the gel was transferred to Immobilon membrane using a Sartoris Dry Blotter, and transfer was assessed by using prestained protein standards (Sigma Chemical Co., NSW Australia). Membranes containing the transferred proteins were blocked with PBS containing either 3% BSA (Sigma, NSW Australia) or 10% dried milk fat powder. After blocking, the membranes were rinsed in PBS/0.5% BSA/0.02% Tween 20 (buffer A) and incubated with a 1:1000 dilution of anti-VEGF antisera (kindly provided by Dr. Trevor Malden, Flinders Medical Center, South Australia, Australia). After an incubation of 1 h at room temperature, excess antibody was removed from the membrane by washing for 30 min in buffer A. Incubation with a 1:2000 dilution of horseradish peroxidase-conjugated, antirabbit immunoglobulin (Bio-Rad, Hercules, CA) followed for an additional 1 h at room temperature. After washing, the bound antibody complexes were detected using an ECL chemiluminescence reagent and XAR film (Kodak), as described by the manufacturers (Amersham).

In Vitro Growth Rate. Rat C6 glioma cells and cells transfected with antisense-VEGF were cultured at 10^4 cells and grown under standard culture conditions. Cell counts were performed on a hemocytometer, initially at 12-h time points, and then every 24 h for a total of at least 160 h. The total number

of cells from duplicate experiments was determined as a function of time (h), and the rate of division was calculated from the exponential phase of growth.

Hypoxic Culture Conditions. Rat C6 glioma cells and the transfected clones were grown in RPMI/5% NCS in 175 cm^2 flasks until 70% confluent. To grow cells under hypoxic conditions, fresh culture medium was added to the flasks, supplemented with $100 \mu\text{M}$ CoCl_2 (28). Conditioned medium was collected from the cells, and total RNA was isolated at 0, 4, 8, and 24 h of culture under hypoxia.

In Vivo Tumor Analysis. Adult male or female nude (*nu/nu*) mice (5–8 weeks of age) received s.c. injections of C6 glioma cells or C6 glioma cells transfected with the antisense-VEGF or vector alone constructs. The mice were anesthetized by methoxyfluorane inhalation. Approximately 10^6 cells resuspended in a volume of $100 \mu\text{l}$ of serum-free cell culture medium were s.c. injected into the flanks of mice. The mice were monitored daily, and the tumor sizes were determined by tridimensional calliper measurements. Tumor volume was calculated as $\text{length} \times \text{width} \times \text{height} (\text{cm}^3)$. Mean tumor volumes were calculated from measurements performed on four mice in each of two individual experiments.

Immunohistochemistry. s.c. tumors were removed from mice and immediately mounted in TissueTEK OCT compound (Miles Inc., Elkhart, IN),

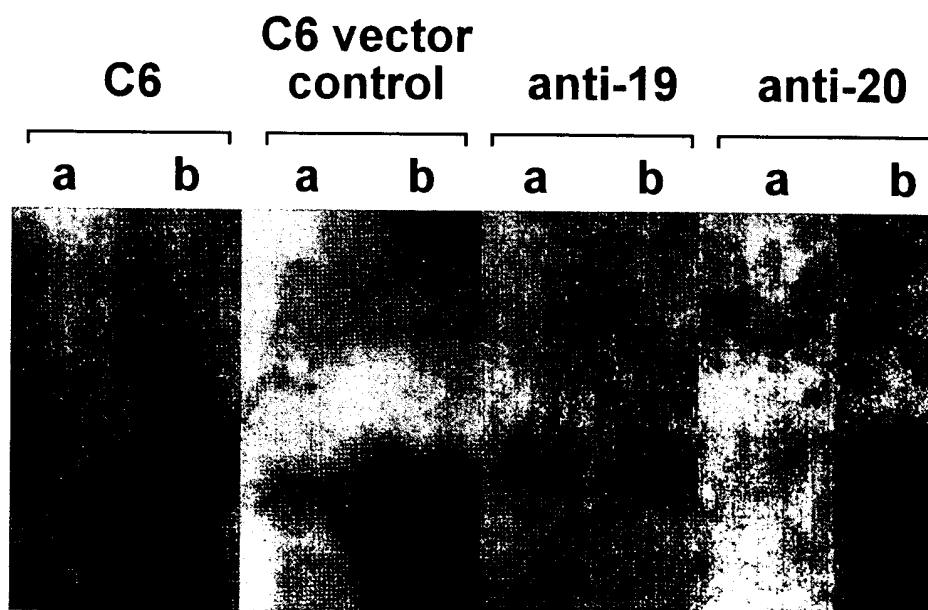


Fig. 4. Western blot analysis of VEGF expression in conditioned medium from cells grown with or without $100 \mu\text{M}$ CoCl_2 to simulate hypoxia and supernatants collected at 0 h (a) and 24 h (b).

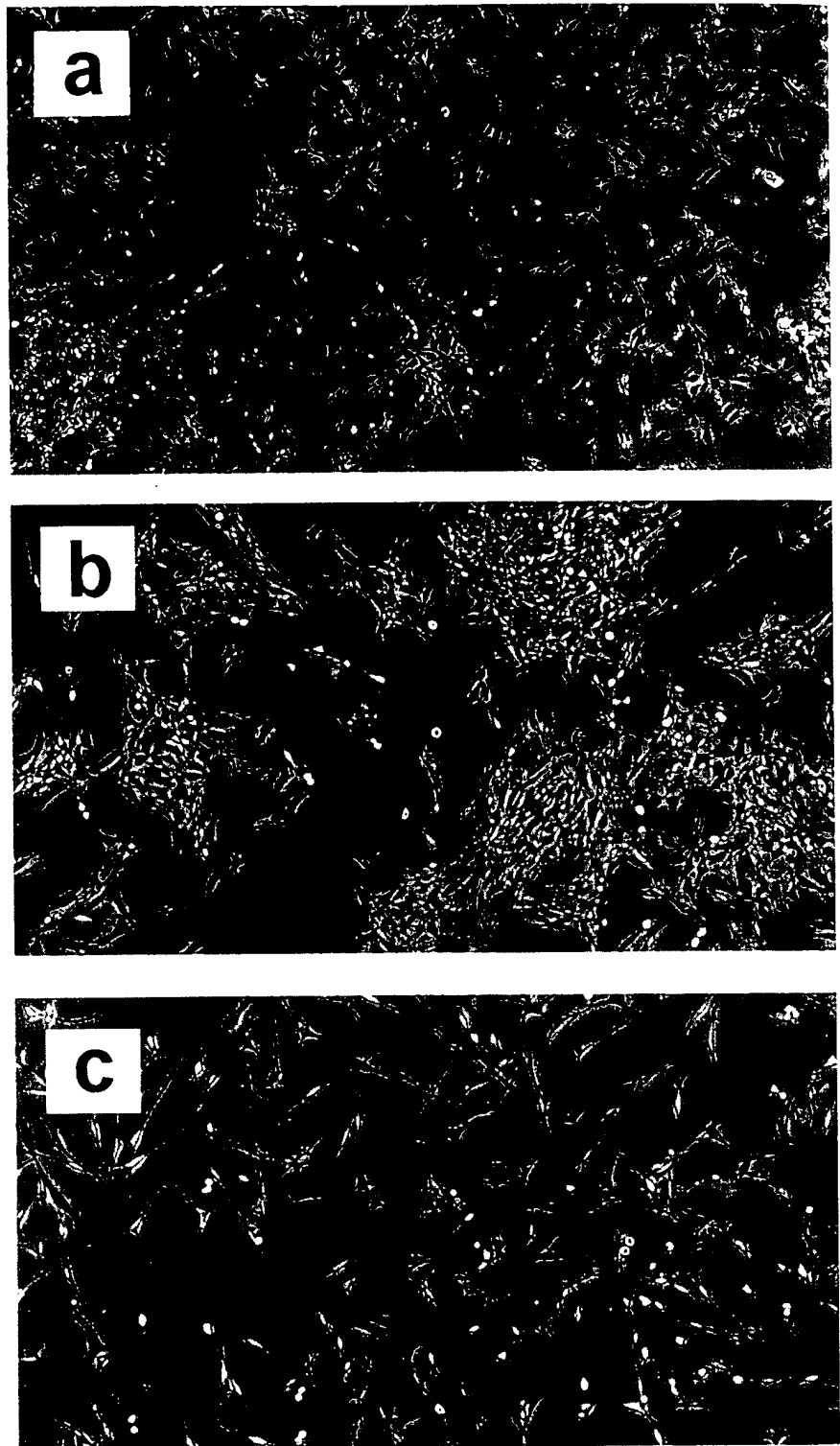


Fig. 5. Photomicrographs of the cell lines cultured under standard conditions as described in "Materials and Methods." *a*, rat C6 glioma; *b*, C6 vector-transfected control; *c*, anti-19 ($\times 40$).

frozen in liquid nitrogen, and stored at -70°C until required. Frozen sections of $7\text{ }\mu\text{m}$ were cut serially through the entire tumor. Alternate sections were stained either with hematoxylin and eosin or by indirect immunoperoxidase with the rat antimouse PECAM monoclonal antibody (PharMingen, San Diego, CA), which detects vascular endothelial cells in the tumors. For immunoperoxidase, sections were fixed in acetone (10 min at -20°C) and rinsed in PBS, as was performed following each of the incubation steps. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxidase. The sections were then incubated with PBS/10% mouse serum for 30 min at room temperature, followed by 1 h at room temperature

with the rat antimouse PECAM monoclonal antibody diluted in PBS/10% mouse serum. A peroxidase-conjugated rabbit antirat immunoglobulin secondary antibody (DAKO, Glostrup, Denmark) diluted in PBS/10% mouse serum was then added to the sections for 30 min at room temperature. The substrate solution of 0.06% (w/v) diaminobenzidine-0.03% (v/v) H_2O_2 in PBS was then added to the sections for 5 min at room temperature. The sections were counterstained with Harris' hematoxylin.

Tumor Necrosis and Vascularization. The percentage of necrosis present in each tumor section was measured using the Flinders MD-30 image analysis system (Leading Edge Technology, South Australia, Australia), and calcula-

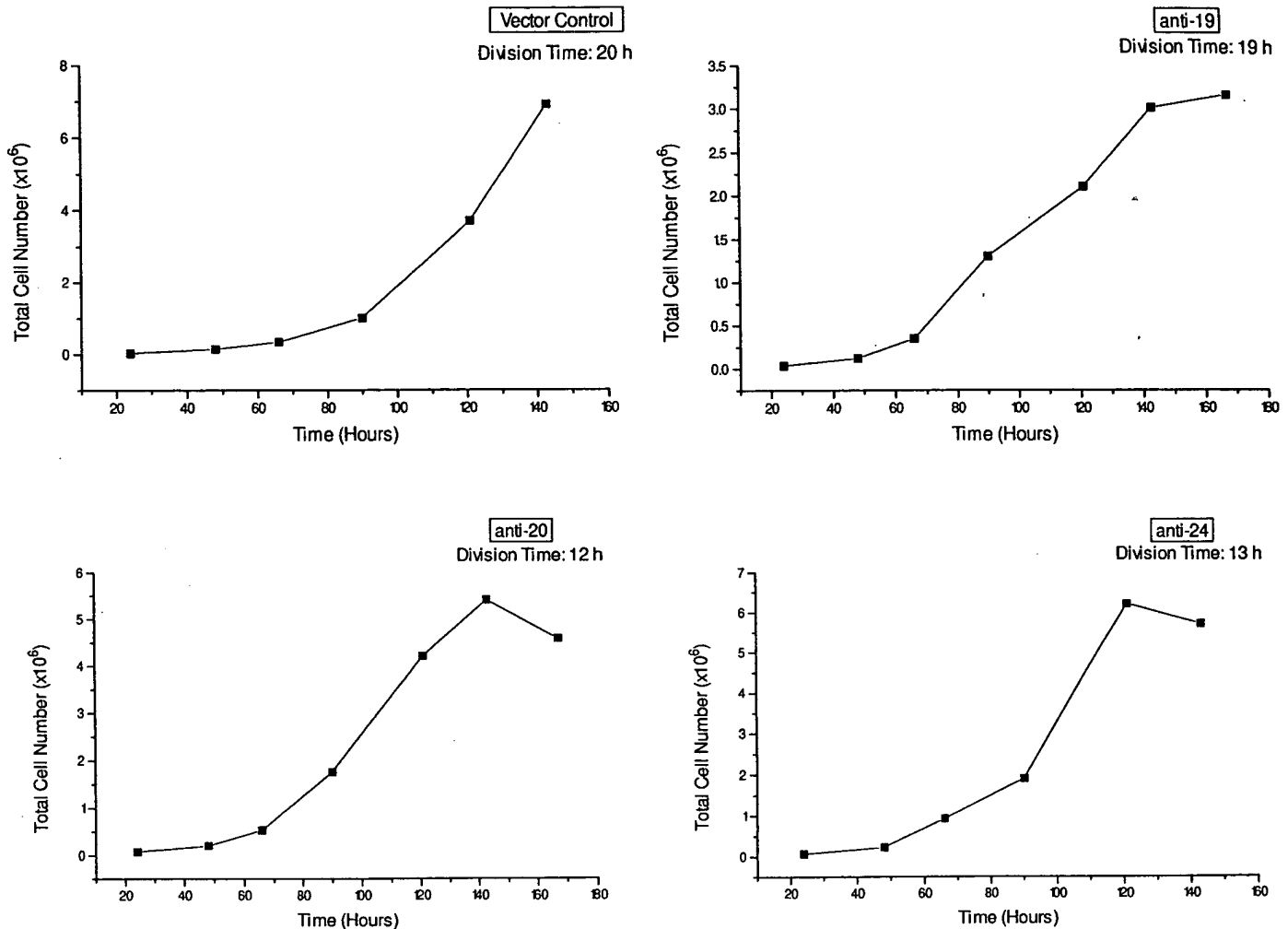


Fig. 6. The growth of C6 glioma control cells and C6 antisense-VEGF cell lines (anti-19, anti-20, and anti-24) as a function of time. Cells were passaged at 10^4 cells (time = 0 h) and allowed to grow under standard culture conditions over a period of 160–180 h. The cell number was determined by hemocytometer counting. The total cell number is presented as the mean of duplicate experiments.

tions were performed by determining the length of bisecting lines (length \times width) through each necrotic site. The sum of the area of the necrotic sites was expressed as the percentage of the area of the tumor sections (length \times width). Calculations were performed on duplicate sections spanning from the outermost surface of each tumor and the middle of each tumor. This was repeated for two tumors/cell line to determine the mean percentage necrosis \pm SE.

The relative number of vascular endothelial cells/tumor section was calculated by counting the number of vessels in a field (0.6×0.4 mm). Calculations were performed on 10 fields selected at random/section. This was performed on five sections/tumor (to determine the mean vessels/field \pm SE) and two tumors/cell line. A Student *t* test (paired, unequal variance) was also used to calculate the level of significance of the tumor growth rates derived from control C6 cells and the antisense-VEGF cell lines. Tumor sections were selected from the outermost edge of the tumor through to the middle and other outer edge to ensure that sampling was throughout the entire tumor.

RESULTS

Cloning of the Antisense-VEGF cDNA. The VEGF insert was cleaved from pBluescript (KS-) by *Xba*I digestion and cloned into the *Xba*I restriction site of the eukaryotic expression vector, pEF-BOS. Restriction enzyme mapping performed on DNA from transformed clones demonstrated the presence of the VEGF₁₆₅ cDNA

insert cloned in the antisense orientation in the pEF-BOS vector (Fig. 1).

Rat C6 Glioma Cells Expressing Antisense-VEGF. Following transfection of rat C6 glioma cells with the antisense-VEGF construct (or vector alone control) and subsequent antibiotic selection, 40 individual clones were isolated and grown in 24-well culture plates. PCR analysis of DNA isolated from these clones revealed that 60% of clones had the antisense-VEGF construct. Of these positive clones, 85% of clones were also shown to express the antisense-VEGF insert as assessed by RT-PCR analysis. A selection of these clones were then re-cloned at the level of one cell/well, and the RT-PCR analysis was repeated to confirm expression of the cDNA. Three antisense-VEGF cell lines were then selected for further analysis and will be referred to as anti-19, anti-20, and anti-24. Three individual clones of C6 cells transfected with vector alone were also analyzed, and the data presented is from vector control clone 8.

Southern blot analysis was performed on genomic DNA of the three antisense-VEGF clones to assess copy numbers of the integrated cDNA (Fig. 2). Genomic DNA was digested with *Eco*RI, and the membranes were hybridized with the VEGF cDNA insert. It was evident that each of the antisense-VEGF cell lines had multiple copies of the insert cDNA, with anti-19 having the highest copy number, followed by anti-24 and then anti-20.

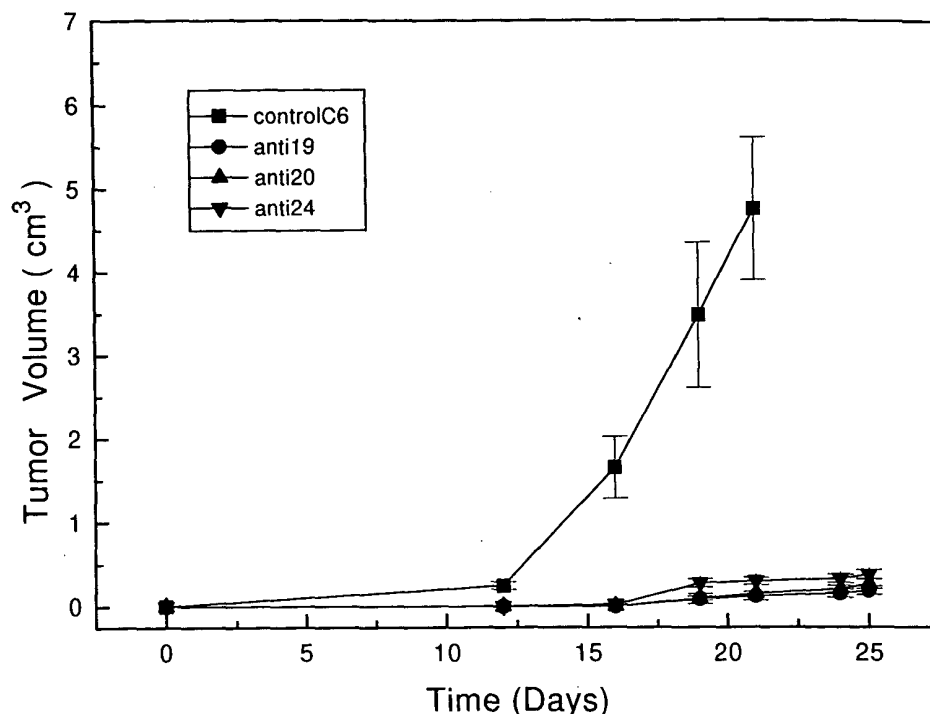


Fig. 7. The growth rate of s.c. tumors implanted in nude mice. Tumor volumes were determined by tridimensional calliper measurements and are presented as the mean tumor volume cm^3 of tumors from four mice in two different experiments; bars, SE.

Response of the Antisense-VEGF Cell Lines to Hypoxia. Control rat C6 glioma cells and transfected cell lines were grown under hypoxic culture conditions, and total RNA was isolated at 0, 4, 8, and 24 h of culture. Northern blot analysis was performed to determine the level of induction of endogenous VEGF expressed in these cell lines (Fig. 3). This experiment also demonstrated the level of expression of the antisense-VEGF cDNA. It is evident that anti-19 expressed antisense-VEGF at a greater level than anti-24, with the lowest level expressed by anti-20 (Fig. 3). Induction of endogenous VEGF expression was observed in normal C6 cells following 24 h of hypoxia (4.2 kb; Fig. 3). However, at this time point, endogenous VEGF mRNA expression was not observed in the anti-19 cell line. After 24 h of hypoxia, endogenous VEGF mRNA was expressed in both anti-20 and anti-24 cell lines, yet at a lower level than in normal C6 cells. An additional product (approximately 2.4 kb) was also observed, with anti-19 and anti-20 clones grown under hypoxia, and may represent splice products. These results were also confirmed by Western blot analysis whereby the conditioned medium of each of the cell lines (C6, C6 vector control, anti-19, and anti-20 at the same time points RNA was extracted) was assessed for VEGF expression (Fig. 4). VEGF was detected in conditioned medium from C6 cells after 24 h of hypoxia, while it remained undetectable in anti-19. There was also detectable VEGF secreted by anti-20, although at a lower level than by C6 cells.

In Vitro Growth Properties of Antisense-VEGF Cell Lines. The antisense-VEGF cell lines appeared phenotypically indistinguishable from normal C6 glioma cells and C6 transfected vector alone cells (Fig. 5). However, the growth rates of the different antisense-VEGF cell lines differed markedly from the control cells (Fig. 6). The division time determined for C6 vector alone control cells was 20 h (Fig. 6), which is identical to that of normal C6 cells (data not shown). The growth rates of three individual clones of the C6 vector control cells were determined and shown to have identical growth patterns (data not shown). Anti-19 cells grew at a similar rate to control cells (19 h). However, the other two antisense-VEGF cell lines grew far more rapidly than controls, with anti-20 and anti-24 having division

times of 12 and 13 h, respectively (Fig. 6). This result was also evident from soft agar colony assays in which anti-19 resulted in a similar number of colonies as control cells, while anti-20 and anti-24 resulted in a 3-fold greater number of colonies (data not shown).

In Vivo Growth of the Antisense-VEGF Cell Lines. Control rat C6 glioma cells and antisense-VEGF cell lines were s.c. injected into nude mice, and tumor volumes were measured daily for the duration of the experiments. The *in vitro* growth rates of the tumors arising from the normal and C6 vector alone control cells were identical; therefore, all of the results shown will be of the vector-alone cells because their growth has not been reported previously, whereas C6 cell growth has been reported by several laboratories. Tumor growth was detectable and measurable for control C6 cells by 12 days postimplantation, while the antisense-VEGF cell lines had yet to give rise to tumors (Fig. 7). Examination of mice at 16 days postimplantation revealed that the control C6 cells had produced tumors of 1.65 ± 0.37 (SE) cm^3 , while none of the antisense-VEGF lines had produced visible tumors. At day 19, the control cells had resulted in tumors of 3.48 ± 0.87 (SE) cm^3 , while the antisense-VEGF cell lines had only just begun to give rise to tumors of 0.08 ± 0.06 (SE) cm^3 , 0.09 ± 0.03 cm^3 , and 0.27 ± 0.05 cm^3 for anti-19, anti-20, and anti-24, respectively. At 21 days postimplantation, the control tumors measured 4.75 ± 0.85 (SE) cm^3 , and the control section of the experiment was terminated. At this time point, however, the tumors from the antisense-VEGF cell lines had not grown significantly from day 19; therefore, the monitoring of the growth of these tumors was continued until day 25. At this time point, the tumors from the antisense-VEGF cell lines had remained at almost the same size as those measured at day 19. Anti-19, anti-20, and anti-24 cell lines produced tumors of 0.18 ± 0.05 (SE) cm^3 , 0.26 ± 0.06 cm^3 , and 0.37 ± 0.06 cm^3 , respectively (Fig. 7).

Tumor Necrosis. Sections of tumors were analyzed for their degree of tissue necrosis. Once again, the vector control C6 cells produced results indistinguishable from normal C6 cells. The results presented in Figs. 8 and 9 demonstrate that there is a higher degree of necrosis in the tumors of the antisense-VEGF cell lines in comparison to tumors produced by control C6 cells. Control C6 tumors had an average necrosis of $9.3 \pm 2.5\%$ (SE) and

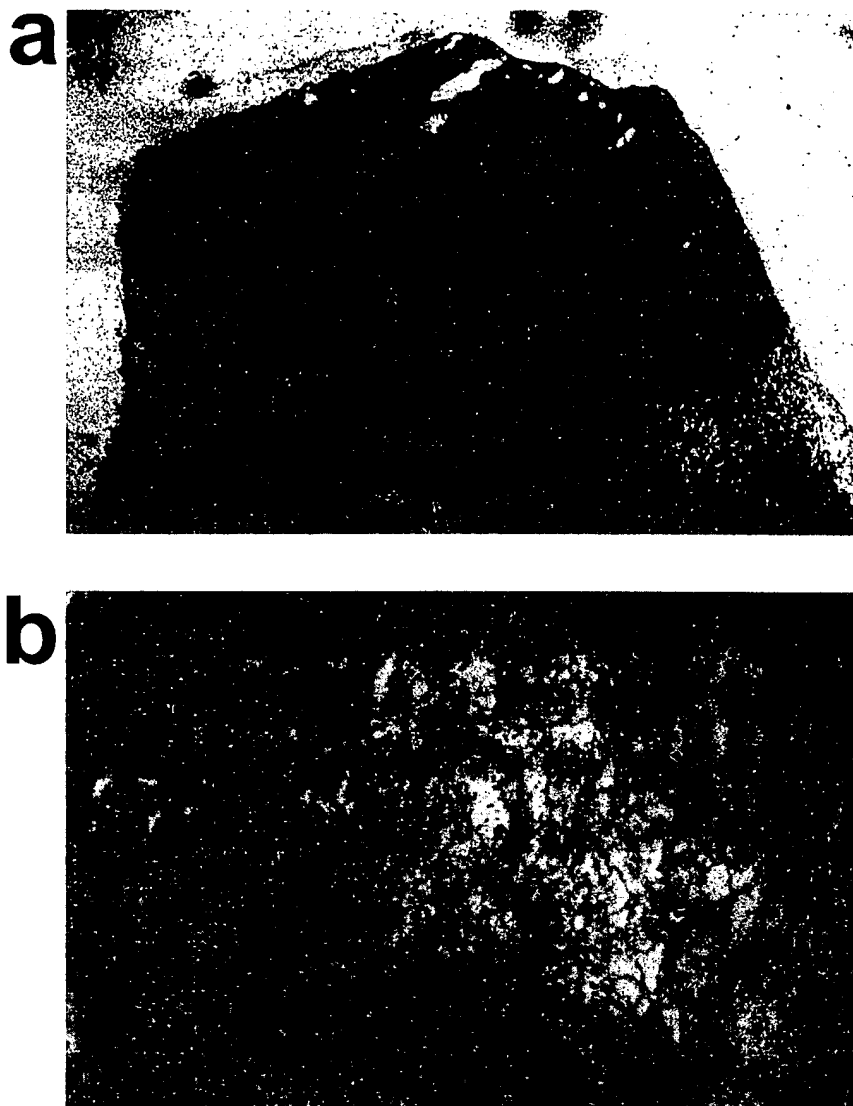


Fig. 8. Photomicrographs of tumor sections stained with hematoxylin and eosin demonstrating an increase in necrosis in the tumors derived from the antisense-VEGF cell lines compared to control C6 glioma cells. *a*, C6 vector control; *b*, anti-19 ($\times 40$).

$6.8 \pm 4.7\%$. In contrast, the antisense-VEGF cell lines were significantly more necrotic, ranging from $14.9 \pm 2.1\%$ (SE) for anti-19 (tumor #3) through to $35.0 \pm 13.2\%$ for anti-20 (tumor #2) (Fig. 9).

Tumor Vascularization. The average number of blood vessels observed in the tumors derived from the antisense-VEGF cells were significantly lower ($P < 0.03$ for each antisense-VEGF cell line compared to control) than in control tumors (Fig. 9 and Fig. 10). Control C6 tumors had an average of $10.3 \pm 1.8\%$ (SE) and $11.2 \pm 1.7\%$ blood vessels/field (0.6×0.4 mm) per tumor (Fig. 10). The number of vessels observed in the antisense-VEGF derived tumors were often up to 50% lower than controls, as observed with anti-19 (tumor #3) and anti-24 (tumor #3). The highest level of vascularization was observed with the anti-20 (tumor #4) tumor, which had an average of 7.5 ± 1.2 vessels/field/tumor. However, this was still a 30% reduction in vessels compared to control tumors. It is important to note that the tumor of anti-20 (tumor #4) was also the least necrotic of the tumors (Fig. 10) and was observed to have grown into adjacent muscle tissue.

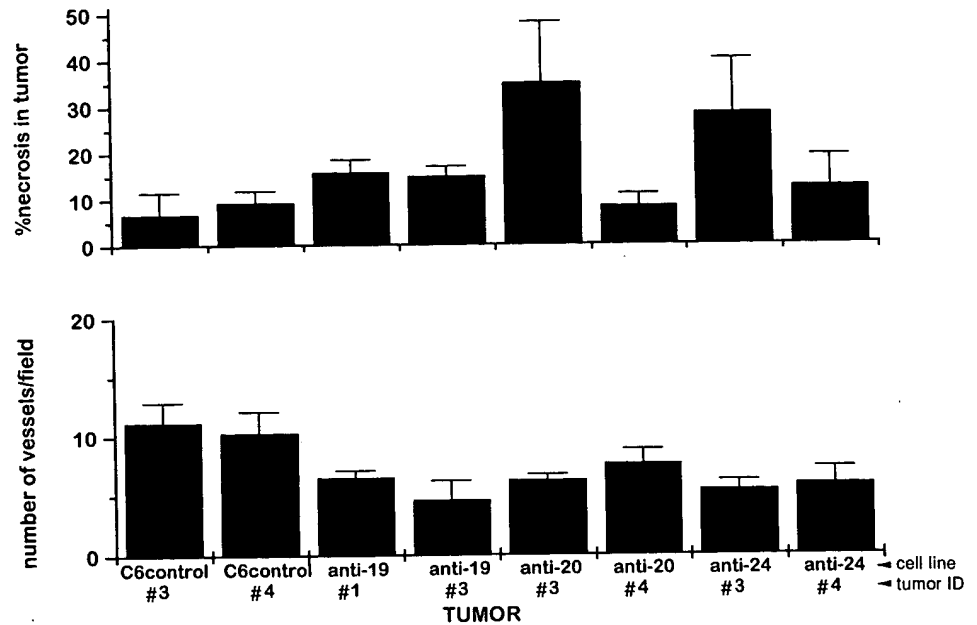
DISCUSSION

It is now evident, as with other cancers, that a number of specific growth factors play significant roles in the promotion of the growth,

progression, and invasive competence of astrocytomas. Thus, while a potential therapy based around the interruption of paracrine and/or autocrine growth factor pathways that impinge upon the tumor cells themselves might well prove to be a successful antitumor approach, it would be difficult to find a pathway that is universally applicable to all tumors, due to the wide variation in the extent to which such growth factor-mediated pathways are important to the growth of such tumors.

Exploiting the ubiquity of tumor angiogenesis as a suitable target for therapy has been proposed previously to be an important concept for antitumor therapy (29). This concept has recently been the subject of renewed interest in the development of new therapeutic strategies. A significant body of evidence is accumulating in favor of the notion that VEGF and its receptors play an important role in the development of solid tumors, such as those derived of glial origin. This pivotal role seems not to be a direct effect upon the tumor cells themselves, but rather it appears to be important to the process of tumor angiogenesis. Three lines of evidence have elegantly demonstrated that this growth factor and its receptors are excellent targets for the development of antitumor strategies based on the inhibition of tumor angiogenesis. These are: the demonstration that VEGF is inducible by rendering cells hypoxic (2, 12–14); the inhibition of *in vivo* tumor growth by antibodies against VEGF (15); and the transient inhibition of tumor

Fig. 9. Analysis of the degree of necrosis and number of blood vessels observed in s.c. tumors of C6 glioma cells and antisense-VEGF cell lines. *Upper panel*, the mean percentage of necrosis determined for each tumor; *bars*, SE. *Lower panel*, the mean number of blood vessels/field (0.6×0.4 mm) for individual tumors derived from each of the cell lines; *bars*, SE. The identification (ID) number of the tumor sample is shown for each of the cell lines examined.



growth by infection of tumor blood vessels with retroviruses bearing dominant-negative mutants of the VEGF-R2 (16) receptor.

In this study, we have added a new dimension to these observations and demonstrated that the inhibition of VEGF expression in the rat

glioma cell line, C6, results not only in the severely impaired growth of these tumors *in vivo* but also in markedly reduced vascularization of the tumors and increased levels of necrosis. The inhibition of tumor growth is even more significant in light of the increased growth rate

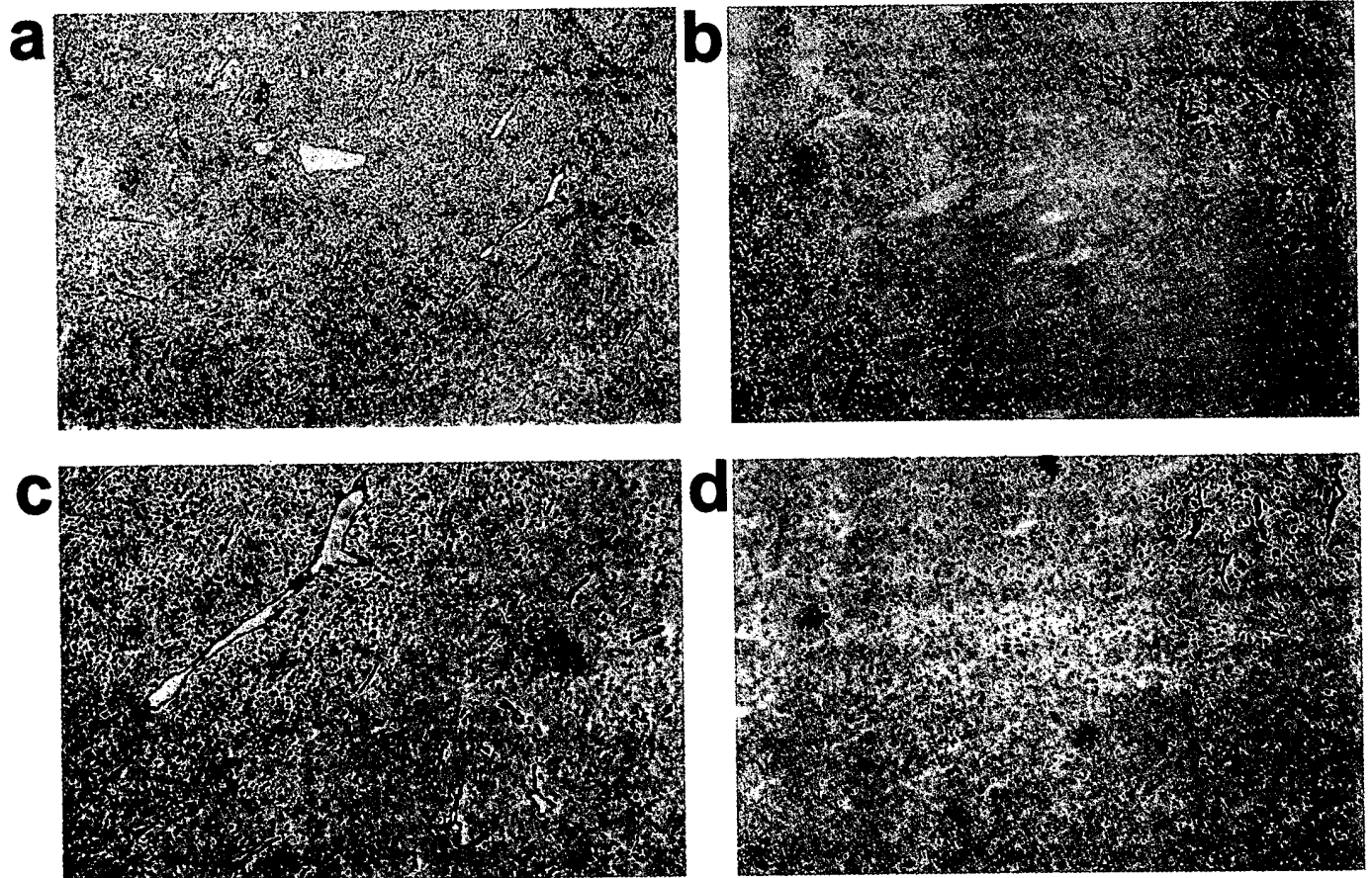


Fig. 10. Photomicrographs of tumor sections stained with a rat antimouse PECAM monoclonal antibody by indirect immunoperoxidase. A diaminobenzidine substrate system was used to visualize antibody binding and is represented by the brown precipitate. Tissue sections were counterstained with Harris' hematoxylin. There is a significant reduction in the number and size of the blood vessels in the tumors of antisense-VEGF cell lines. *a*, C6 control cells ($\times 40$); *b*, anti-19 ($\times 40$); *c*, C6 control cells ($\times 100$); *d*, anti-19 ($\times 100$).

of two of these antisense-VEGF cell lines *in vitro*. Furthermore, the reduced levels of VEGF produced by the antisense-VEGF-transfected C6 cell lines resulted in a decrease of both the number and the thickness of tumor blood vessel walls, while levels of necrosis, and presumably the various metabolites released by necrotic tissues, were significantly increased. This observation suggests that VEGF is the principle mediator of tumor angiogenesis and that the absence of a VEGF response cannot be adequately compensated for by other angiogenic factors, such as basic fibroblast growth factor or PDGF. Interestingly, much of the angiogenesis that was observed in the tumors derived from the antisense-VEGF-transfected C6 cells appeared to be located in small clusters of vessels, which may have been derived from one or a small number of vessels that had locally escaped the suppression of VEGF production. Whether these clusters of vessels indicated the presence of clones of C6 cells that had lost the ability to produce the antisense transcript and had, therefore, regained the capacity to recruit new blood vessels, could not be judged in this study.

It has been reported recently that VEGF suppresses the cell surface expression of E-selectin and V-CAM on endothelial cells both *in vivo* and *in vitro* (30). It might be anticipated, therefore, that a diminution of eosinophil adherence (*inter alia*) to these endothelial cells would result from the tumor-associated production of VEGF. Our preliminary observations assessing the degree of eosinophil infiltration of both control C6 and antisense-VEGF-transfected tumors (data not shown) fail to support this notion, and it seems unlikely that the production of VEGF is a strategy by which the tumors seek to avoid destruction by the host immune system. Rather, we favor the notion that VEGF is the primary motive force for the recruitment of blood vessels in tumors, at least in this model system.

Intriguingly, our data also hint at the possibility that VEGF may have an autocrine effect upon the C6 cells themselves. We have demonstrated that expression of antisense-VEGF results in a halving of cell division times *versus* control vector-transfected cells, suggesting that the VEGF expressed by C6 may be inhibiting their growth. As individual clones of vector control cells were analyzed, the difference in growth rates cannot be attributed to clonal variation within the original C6 cells. There is no evidence at present to suggest that C6 cells possess receptors for VEGF. Indeed, data from other laboratories suggest that C6 do not express either VEGF-R1 or VEGF-R2 (13, 31). Moreover, the expression of VEGF-R1 and VEGF-R2 has been perceived to be the sole domain of endothelial cells and their precursors; thus, our observations will require further work to establish the possible involvement of a VEGF autocrine loop in the C6 glioma system. However, our findings do demonstrate that the inhibition of VEGF is sufficient to control tumor growth *in vivo* by the suppression of tumor neovascularization. The antisense-VEGF strategy offers a new avenue of gene therapy development as an adjuvant treatment for human glioma.

ACKNOWLEDGMENTS

We thank Professor A. H. Kaye for his continuing support and discussion, and Stan S. Stylli and K. Vasilopoulos for their technical assistance.

REFERENCES

- Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* (Washington DC), 246: 1306-1309, 1989.
- Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* (Lond.), 359: 843-845, 1992.
- Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* (Washington DC), 246: 1309-1312, 1989.
- Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.*, 5: 1806-1814, 1991.
- De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* (Washington DC), 255: 989-991, 1992.
- Millauer, B., Witzigmann Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ullrich, A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*, 72: 835-846, 1993.
- Oelrichs, R. B., Reid, H. H., Bernard, O., Ziemiecki, A., and Wilks, A. F. NYK/FLK-1: a putative receptor protein tyrosine kinase isolated from E10 embryonic neuroepithelium is expressed in endothelial cells of the developing embryo. *Oncogene*, 8: 11-18, 1993.
- Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.*, 187: 1579-1586, 1993.
- Tischer, E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J. C., and Abraham, J. A. Vascular endothelial growth factor: a new member of the platelet-derived growth factor gene family. *Biochem. Biophys. Res. Commun.*, 165: 1198-1206, 1989.
- Conn, G., Bayne, M. L., Soderman, D. D., Kwok, P. W., Sullivan, K. A., Palisi, T. M., Hope, D. A., and Thomas, K. A. Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA*, 87: 2628-2632, 1990.
- Breier, G., Albrecht, U., Sterrer, S., and Risau, W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* (Camb.), 114: 521-532, 1992.
- Plate, K. H., Breier, G., Weich, H. A., and Risau, W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* (Lond.), 359: 845-848, 1992.
- Plate, K. H., Breier, G., Weich, H. A., and Risau, W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* (Lond.), 359: 845-848, 1993.
- Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* (Lond.), 359: 843-845, 1993.
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* (Lond.), 362: 841-844, 1993.
- Millauer, B., Shawver, L. K., Plate, K. L., Risau, W., and Ullrich, A. Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. *Nature* (Lond.), 367: 576-579, 1994.
- Kaye, A. H., and Hill, J. S. Photodynamic therapy of brain tumors. *Ann. Acad. Med. Singapore*, 22: 470-481, 1993.
- Woodburn, K. W., Hill, J. S., Stylli, S., Kaye, A. H., Reiss, J. A., and Phillips, D. R. Evaluation of a morpholinothiopyrrole for use in photodynamic therapy. *Br. J. Cancer*, 70: 398-400, 1994.
- Gillaspay, G. E., Mapstone, T. B., Samols, D., and Goldthwait, D. A. Transcriptional patterns of growth factors and proto-oncogenes in human glioblastomas and normal glial cells. *Cancer Lett.*, 65: 55-60, 1992.
- Pedersen, P., Ness, G. O., Engerbraaten, O., Bjerkvig, R., Lillehaug, J. H., and Laerum, O. D. Heterogeneous response to the growth factors [EGF, PDGF (bb), TGF- α , bFGF, IL-2] on glioma spheroid growth: migration and invasion. *Int. J. Cancer*, 56: 255-261, 1994.
- Brem, S., Cotran, R., and Folkman, J. Tumour angiogenesis: a quantitative method for histological grading. *J. Natl. Cancer Inst.*, 48: 347-356, 1972.
- Kaye, A. H., Morstyn, G., Gardner, I., and Pyke, K. Development of a xenograft glioma model in mouse brain. *Cancer Res.*, 46: 1367-1373, 1986.
- Mizushima, S., and Nagata, S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, 18: 5322, 1990.
- Graham, F. L., and Eb, A. J. V. Transformation of rat cells by DNA of human adenovirus 5. *Virology*, 54: 536-539, 1973.
- Church, G. M., and Gilbert, W. Genomic Sequencing. *Proc. Natl. Acad. Sci. USA*, 81: 1991-1995, 1984.
- Feinberg, A. P., and Vogelstein, B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 137: 266-267, 1984.
- Chirgwin, J. M., Przybyla, A. E., McDonald, F. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, 18: 5294-5299, 1979.
- Minchenko, A., Salceda, S., Bauer, T., and Caro, J. Hypoxia regulatory elements of the human vascular endothelial growth factor gene. *Cell. Mol. Biol. Res.*, 40: 35-39, 1994.
- Folkman, J. Anti-angiogenesis: new concepts of therapy of solid tumors. *Ann. Surg.*, 175: 409-416, 1972.
- Roberts, G., and Bevilacqua, M. P. Vascular endothelial growth factor inhibits E-selectin and VCAM expression *in vitro* and *in vivo*. *J. Cell. Biochem. Suppl.*, 18A: E2312, 1994.
- Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Senger, D. R., and Dvorak, H. F. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res.*, 53: 4727-4735, 1993.